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The effects of 4-Phenylbutyric acid on ER stress during mouse tooth development

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Introduction: During tooth development, proper protein folding and trafficking are significant processes as newly synthesized proteins proceed to form designated tissues. Endoplasmic reticulum (ER) stress occurs inevitably in tooth development as unfolded and misfolded proteins accumulate in ER. 4-Phenylbutyric acid (4PBA) is a FDA approved drug and known as a chemical chaperone which alleviates the ER stress. Recently, several studies showed that 4PBA performs therapeutic effects in some genetic diseases due to misfolding of proteins, metabolic related-diseases and apoptosis due to ER stress. However, the roles of 4PBA during odontogenesis are not elucidated. This study revealed the effects of 4PBA during molar development in mice.

Methods: We employed in vitro organ cultivation and renal transplantation methods which would mimic the permanent tooth development in an infant period of human. The *in vitro* cultivated tooth germs and renal calcified teeth were examined by histology and immunohistochemical analysis.

Results and Discussion: Our results revealed that treatment of 4PBA altered expression patterns of enamel knot related signaling molecules, and consequently affected cellular secretion and patterned formation of dental hard tissues including dentin and enamel during tooth morphogenesis. The alteration of ER stress by 4PBA treatment during organogenesis would suggest that proper ER stress is important for pattern formation during tooth development and morphogenesis, and 4PBA as a chemical chaperone would be one of the candidate molecules for dental and hard tissue regeneration.

KEYWORDS

chemical chaperones, dentinogenesis, morphogenesis, secretion, signaling

Introduction

Tooth development starts from thickening of oral epithelium followed by the migration of neural crest-derived mesenchyme below the epithelium (Jussila and Thesleff, 2012; Balic, 2018). Interactions between epithelium and the underlying mesenchyme play important roles for development of tooth from bud, cap, bell to secretory stages (Santosh and Jones, 2014; Balic, 2018). At cap stage, enamel knot (EK), a non-proliferating signaling center of tooth development closely linked with epithelial-mesenchymal interactions, expresses important signaling molecules including autocrine and paracrine factors, and transcription factors for proper tooth morphogenesis (Yu and Klein, 2020; Abramyan et al., 2021). At the secretory stage, inner enamel epithelium (IEE) begins differentiate into ameloblasts and the underlying mesenchymal dental papilla (DP) differentiate into odontoblasts for the secretion of enamel and dentin respectively (Otsu et al., 2011; Kawashima and Okiji, 2016). However, when there is disturbance in the secretion of extracellular matrix (ECM) proteins, it may lead to Amelogenesis Imperfecta (AI) or Dentinogenesis Imperfecta (DI) (Smith et al., 2017). It is suggested that AI would be one of the results of ER stress which arises when secreting cells such as ameloblasts have inadvertent stress (Brookes et al., 2014, 2017; Aryal et al., 2019).

The regulation of ER stress is important for proper modulation of cell signaling and secretion during tooth development (Kim J. W. et al., 2014; Brookes et al., 2014, 2017; Aryal et al., 2020). ER stress occurs when the capacity of protein-folding of ER is exceeded (Tabas and Ron, 2011). As ER stress occurs, it induces the phenomenon called unfolded protein response (UPR) in order to maintain protein homeostasis through collection of intracellular signal transduction reactions (Sano and Reed, 2013). During ER stress regulation, the binding immunoglobulin protein (BiP) binds the unfolded proteins in the ER lumen for proper folding of unfolded and misfolded proteins (Pobre et al., 2019). ER stress triggers accumulation of unfolded proteins which leads to nutrient deprivation, hypoxia and point mutation (Zhang et al., 2010; Oakes and Papa, 2015). Recent studies in ER stress revealed that ER stress modulation plays an important role in hard tissue formation, especially dentin formation (Brookes et al., 2014, 2017; Aryal et al., 2019).

The chemical chaperones correct the folding and assemblance of proteins, thus controlling protein trafficking by preventing their degradation or aggregation for proper protein function to alleviate ER stress (Kim et al., 2013; Cortez and Sim 2014). Because of their proteostatic function, it draws increasing attention for many therapeutic agents. Phenylbutric acid (4PBA) is a chemical chaperone in the ER which enhances the capacity of protein folding and also facilitates the proper folding of mutant proteins (Iannitti and Palmieri, 2011; Kim et al., 2013). The clinical use of 4PBA as an ammonia scavenger has been approved (Uppala et al., 2017). In addition, some studies proved the efficacy of 4PBA in metabolic diseases such as obesity and diabetes and in inflammatory diseases (Luo et al., 2010; Kim et al., 2013; Chen et al., 2016; Wu et al., 2020). However, the effects of 4PBA during dodntogenesis has not yet elucidated. Based on our previous study, which revealed the expression patterns of ER stress related signaling molecules during various stages of tooth development (Aryal et al., 2020), thus, we designed to examine the role of 4PBA in tooth development using *in vitro* organ cultivation system.

Materials and methods

The animal experiments performed in this study were ethically approved (KNU 2020-0107). We followed the guidelines from Intramural Animal Use and Care Committee, Kyungpook National University, School of Dentistry.

Animals

The healthy time-mated pregnant ICR mice at embryonic stage 14 (E14) were obtained for embryo collection. Similarly, adult ICR 7-week old male mice were used for renal transplantation experiment. Mice were housed in optimum conditions, including room temperature (22°C ± 2°C), 55 ± 5% humidity and artificial illumination with lights on from 05:00 to 17:00 h, with access to food and water *ad libitum*.

In vitro organ cultivation and drug treatment

Tooth germs at E14 stage were micro-dissected in PBS and then cultivated with either 100 μM 4PBA (Sigma Aldrich, St. Louis, Missouri, United States) or 0.07% DMSO with DMEM (HyClone, Logan, UT, United States; cat. no.-SH30243.01) and 10% fetal bovine serum (Hyclone, Logan, UT, United States) for 48 h using a modified Trowell's culture method as previously described (Kim et al., 2009). The cultured tooth germs were then transplanted into the kidney capsule of 7 week old male mice for one and 3 weeks to obtain the calcified teeth as described previously (Neupane et al., 2020). The harvested renal calcified teeth were photographed using a Leica MZ16FA.

Histology and immunohistochemistry

The *in vitro* cultivated tooth germs and renal calcified teeth were sectioned for histology and immunohistochemical analysis. Before sectioning, the one and 3 week calcified teeth were decalcified using 0.5 M EDTA. The histology and immunohistochemistry (IHC) were performed as described previously (Neupane et al., 2020). For histological analysis, Hematoxylin and eosin (H&E) staining and Masson's trichrome (MTC) staining were performed. Primary

antibodies used in this study are Nestin (Abcam, Cambridge, United Kingdom, 1:400; cat. no. ab11306), Amelogenin (AMELX; Abcam, Cambridge, United Kingdom, 1:500; cat. no. ab153915), Glucose regulatory protein 78 (GRP78; Abcam, Cambridge, United Kingdom, 1:400; cat. no. ab21685) and HRD1 (Novus Biologicals, Colorado, United States, 1:200; cat. no. NB100-2526) and Ki67 (Thermo Scientific, Waltham, MA, United States, 1:400; cat. no. RM-9106-s). 1X western blocking solution (Roche, Mannheim, Germany; Ref. 11921673001) was treated for blocking of non-specific binding of antibody. Similarly, the biotinylated anti-rabbit or anti-rat IgG were used as secondary antibodies. The antibody binding was visualized using the diaminobenzidine tetrahydrochloride reagent kit (GBI Labs, Bothell, WA, United States, cat no. C09-12). The images were photographed using DM2500 microscope (Leica, Wetzlar, Germany).

Quantitative PCR (qPCR)

RNA extraction from *in vitro* cultivated tooth germs and cDNA synthesis for qPCR analysis were performed as described previously (Aryal et al., 2020). The data of real time PCR were normalized to hypoxanthine-guanine phosphoribosyltransferase (Hprt) gene and are expressed as mean ± standard deviation. The primers used during qPCR experiment are presented inSupplementary Table S1.

TUNEL assay

TUNEL assay was performed using *in situ* cell apoptosis detection kit (Trevigen, Gaithersburg, MD, United States, cat. no. 4810-30-K) as described previously (Neupane et al., 2014). The images were photographed using DM2500 microscope (Leica, Wetzlar, Germany).

In situ hybridization

To examine the expression patterns of genes, whole mount and section *in situ* hybridizations were performed in the *in vitro* cultivated tooth germs as described previously (Sohn et al., 2012). For hybridization, digoxigenin-labeled mRNA probes were hybridized overnight at 65°C. The images were photographed using MZ16FA and DM2500 microscope (Leica, Wetzlar, Germany).

Three-dimensional (3D) reconstruction

For 3D reconstruction, the serial sections of cultivated tooth with thickness of 7 μm were photographed. The tooth germs were then reconstructed for each specimen using "Voloom 2.3" software (Micro Dimensions, Germany). The images were then aligned automatically and manually.

Statistical analysis

ImageJ software (http://imagej.net/) was used to count the immunostaining and TUNEL positive cells as described previously (Neupane et al., 2020). The number of Ki67 and TUNEL positive cells in the DAB-stained sections were counted in the defined area of 50 × 50 μm^2. The number of Ki67 positive cells were counted from the epithelium and adjacent mesenchyme, whereas the TUNEL positive cells were counted from the enamel knot area. Similarly, the cusp, crown, root and mesiodistal length of renal calcified teeth were measured using ImageJ (http://imagej.net/) as described previously (Neupane et al., 2020). Data were represented as ± standard deviations and the mean was determined by comparing control and 4PBA treated groups using Student's t-test. $p < .05$ indicates significance.

Results

ER stress in the developing tooth

The normal developing tooth at E14 and E16 represent cap and bell stage respectively (Figures 1A, B). Similar to previous report (Aryal et al., 2 020), the localizations of ER stress related markers: 74-kDa glucose-regulated protein (GRP78) and HMG-CoA reductase degradation protein 1 (HRD1) were observed in the cap and bell stage of tooth development (Figures 1C–F). GRP78, a major ER chaperone protein and a master regulator for ER stress, localized at DP, EK and IEE at cap stage (Figure 1C). However, at bell stage, the localization of GRP78 was restricted only to enamel forming area (Figure 1D). Similarly, HMG-CoA, a key enzyme for ER-associated degradation (ERAD) of misfolded proteins, was localized along the entire tooth germ during cap stage (Figure 1E), whereas, strong localization was observed along the enamel forming area during bell stage (Figure 1F).

Altered cellular physiology after 4PBA treatment

To examine the effect of 4PBA in the developing tooth germ, we performed *in vitro* organ cultivation of E14 tooth germs for 2 days. Cellular proliferation were examined by immunostaining against Ki67, and the apoptosis were examined by TUNEL assay (Figures 2A–F). The number of proliferative cells were decreased in the 4PBA treated groups when compared to control (Figures 2C, D, G). Particularly, the EK area showed decreasing number of

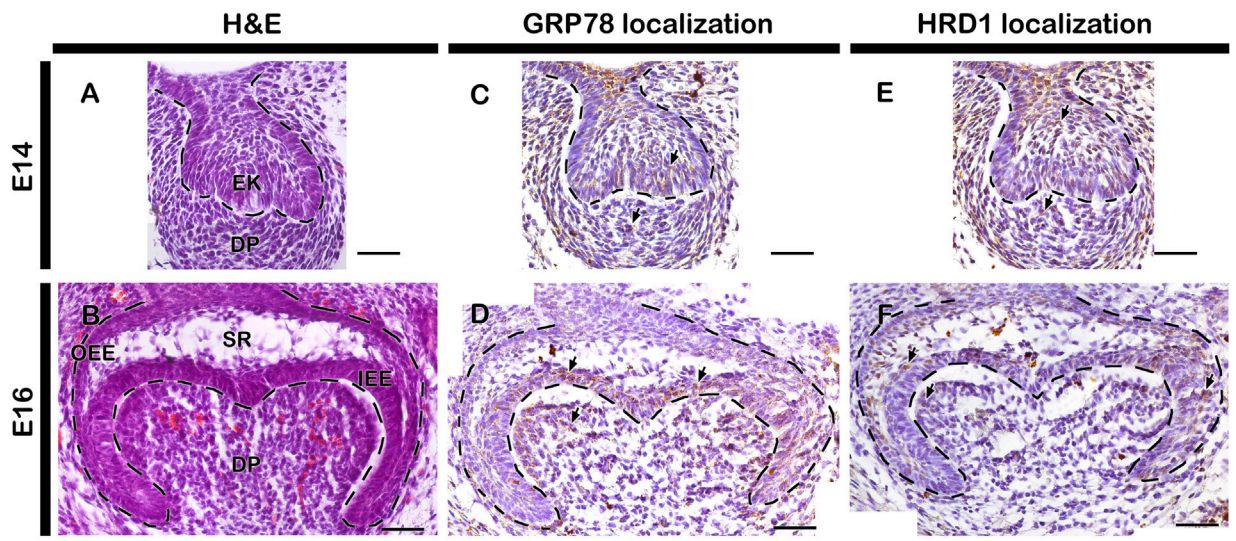

FIGURE 1
Histology and localization patterns of ER stress markers in mouse developing teeth. HE staining showing embryonic tooth germs at cap **(A)** and bell stage **(B)** of tooth development. Localizations of GRP78 and HRD1 at E14 and E16 tooth germs and counterstained with hematoxylin **(C–F)**. Dotted lines demarcate epithelial boundary **(A–F)**. Arrows indicate immunostaining positive cells. EK, enamel knot; IEE, inner enamel epithelium; OEE, outer enamel epithelium; DP, dental papilla; SR, stellate reticulum. Scale bars: 200 μm **(A,C and E)**, 50 μm **(B,D and F)**.

proliferative cells in the 4PBA treated group (Figures 2A–D). Whereas, apoptotic event of 4PBA treated group showed more apoptotic cells, especially at the EK area when compared to control (Figures 2E, F, H). To compare the exact size of EK, the expression pattern of Fgf4 was examined using whole mount *in situ* hybridization, however, there was no obvious differences between control and 4PBA treated specimens (Supplementary Figure S1), To examine the activity of ER stress during *in vitro* cultivation, we performed immunostainings against GRP78 and HRD1 (Figures 3A–D). The localization of GRP78 in the 4PBA treated group was more intense than those of controls throughout the entire tooth germ (Figures 3 A, B, Supplementary Figure S2). Similarly, the localization of HRD1 was stronger along the IEE and dental papilla in the 4 PBA treated tooth germs when compared to control (Figures 3 C, D, Supplementary Figure S2). We also measured the relative expression levels of signaling molecules related to amelogenesis, odontogenesis and ER stress after 24 h of *in vitro* organ cultivation at E14 with treatment of 4PBA. After 12 h of cultivation, all the molecules were up-regulated with treatment of 4PBA, however, their expressions levels did not altered significantly after 24 h (data not shown).

Tooth phenotype after 4PBA treatment

To examine the long term effect of 4PBA in tooth phenotype, we performed kidney capsule transplantation for one and 3 week after *in vitro* cultivation of tooth germs at E14 for 2 days. One week renal calcified teeth displayed denser alignment of odontoblasts with thicker dentin and enamel space in the 4PBA treated teeth when compared to control (Figures 4A, A′, B, B′). Meanwhile, the activations of odontoblasts and ameloblasts were examined with immunostainings against NESTIN and AMELOGENIN (Figures 4C, C′–F, F′). 4PBA treated group showed apparently stronger localization pattern of NESTIN in active odontoblasts when compared to control group (Figures 4C, C′, D, D′). Similarly, strong localization pattern of AMELOGENIN in the 4PBA treated group was observed (Figures 4E, E′, F, F′). Moreover, 3 weeks renal calcified teeth showed altered morphological changes in the 4PBA treated teeth (Figures 5A–D). Particularly, thicker predentin was observed along the cuspal area in the 4PBA treated teeth when compared to control (Figures 5A–D). To compare the control and 4PBA treated teeth precisely, we randomly selected 5 teeth from both groups and measured the cusp, crown, root and mesio-distal length. Interestingly, 4PBA treated group showed shorter cusp and crown length, however, the root and mesio-distal lengths were longer when compared to control (Figure 5E). Especially, root length of 4PBA treated tooth was almost double than that of control group (Figure 5E).

Discussion

ER stress pathway is involved during development and differentiation of hard tissue formation (Cameron et al., 2015; Li et al., 2018; Chen et al., 2020). Especially in tooth development,

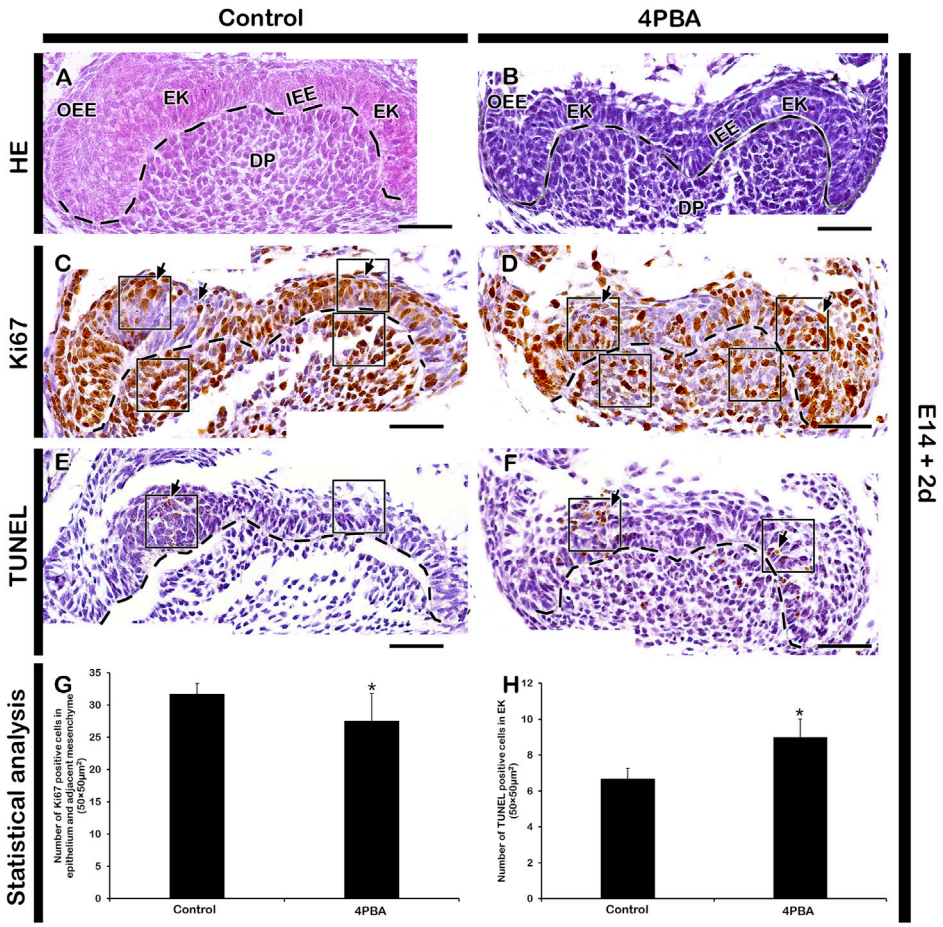

FIGURE 2
Histology, immunostaining of Ki67 and TUNEL assay of 2 days *in vitro* organ cultivated teeth. HE staining of *in vitro* cultivated teeth **(A,B)**. Ki67 immunostaining **(C,D)** and TUNEL assay **(E,F)** in the control and 4PBA treated group. In the 4PBA treated group, proliferating cells are reduced in the epithelium and adjacent mesenchyme **(C,D)**, however, number of apoptotic cells are increased in epithelium when compared to control (E-F). Tissues are counterstained with hematoxylin **(C–F)**. Statistical analysis showing Ki67 and TUNEL positive cells **(G,H)**. N = 6. * indicates $p < .05$ **(G,H)**. Dotted lines demarcate the epithelial boundary **(A–F)**. Arrows indicate immunostaining and TUNEL positive cells **(E,F)**. Square boxes indicate area of 50 $\mu m^2 \times 50\ \mu m^2$ for positive cell count **(E,F)**. EK, enamel knot; SR, stellate reticulum; IEE, inner enamel epithelium; DP, dental papilla. Scale bars: 50 μm **(A–F)**.

ER stress plays a key role in the ameloblast (Brookes et al., 2014; Suzuki et al., 2017; Aryal et al., 2019, 2020) and odontoblast (Kim Y.-G. et al., 2014) differentiation. In addition, ER stress molecules play a vital role in maintaining cellular homeostasis during odontogenesis because their expression patterns coincide with the novel transcription-regulating signaling molecules (Uchibe et al., 2012; Aryal et al., 2020). Particularly, the role of UPR in cap stage is not only alleviating protein overload in the ER, but also interacting with signaling molecules for cellular homeostasis during cap to bell stage transition (Aryal et al., 2020). In addition, the expressions of ER stress regulator genes: Ire1 and Xbp1 at IEE and dental papilla during bell stage suggest that Ire1/Xbp1 ER stress pathway initiates the ameloblast precursor cells to secrete ECM and dental papilla cells to differentiate into odontoblasts beforehand (Tsuchiya et al., 2008; Brookes et al., 2014; Kawashima and Okiji, 2016; Aryal et al., 2020), thereby emphasizing the importance of ER stress molecules and chemical chaperones during extracellular matrix secretion and tooth morphogenesis. Therefore, this study examined the effects of chemical chaperone, 4PBA, during tooth development using *in vitro* organ cultivation system.

The clinical trials of 4PBA in both *in vitro* and *in vivo* as one of the potential therapeutic agent for metabolic diseases such as obesity and diabetes, inflammatory disorders and cancers has been studied (Luo et al., 2010; Iannitti and Palmieri, 2011; Kim et al., 2013; Chen et al., 2016). Especially, 4PBA rescued malformed tooth enamel, mirroring human AI and improved the gross appearance of incisor teeth of (Barron et al., 2010; Brookes et al., 2014). According to Brookes et al., 4PBA does not directly interact with secretory load of ER to restore normal

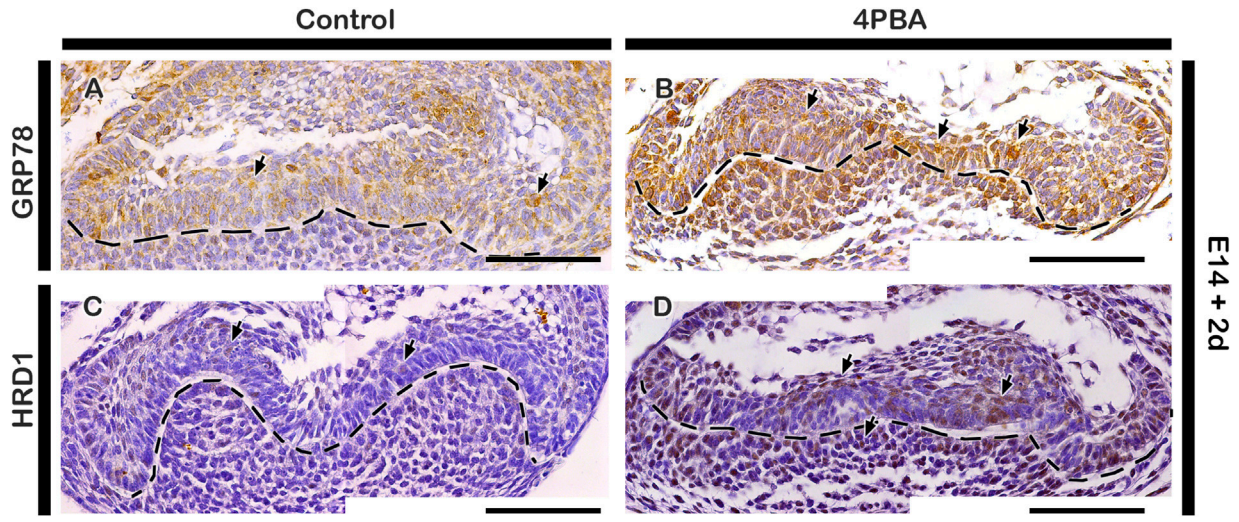

FIGURE 3
Localization patterns of GRP78 and HRD1. *In vitro* organ cultivation at E14 for 2 days showing increased localizations of GRP78 and HRD1 in both epithelium and mesenchyme of 4PBA treated teeth germs compared to controls **(A–D)**. Tissues are counterstained with hematoxylin **(A–D)**. Arrows indicate immunostaining and TUNEL positive cells **(A–D)**. Dotted lines demarcate the epithelial boundary **(A–D)**. Scale bars: 50 μm **(A–D)**.

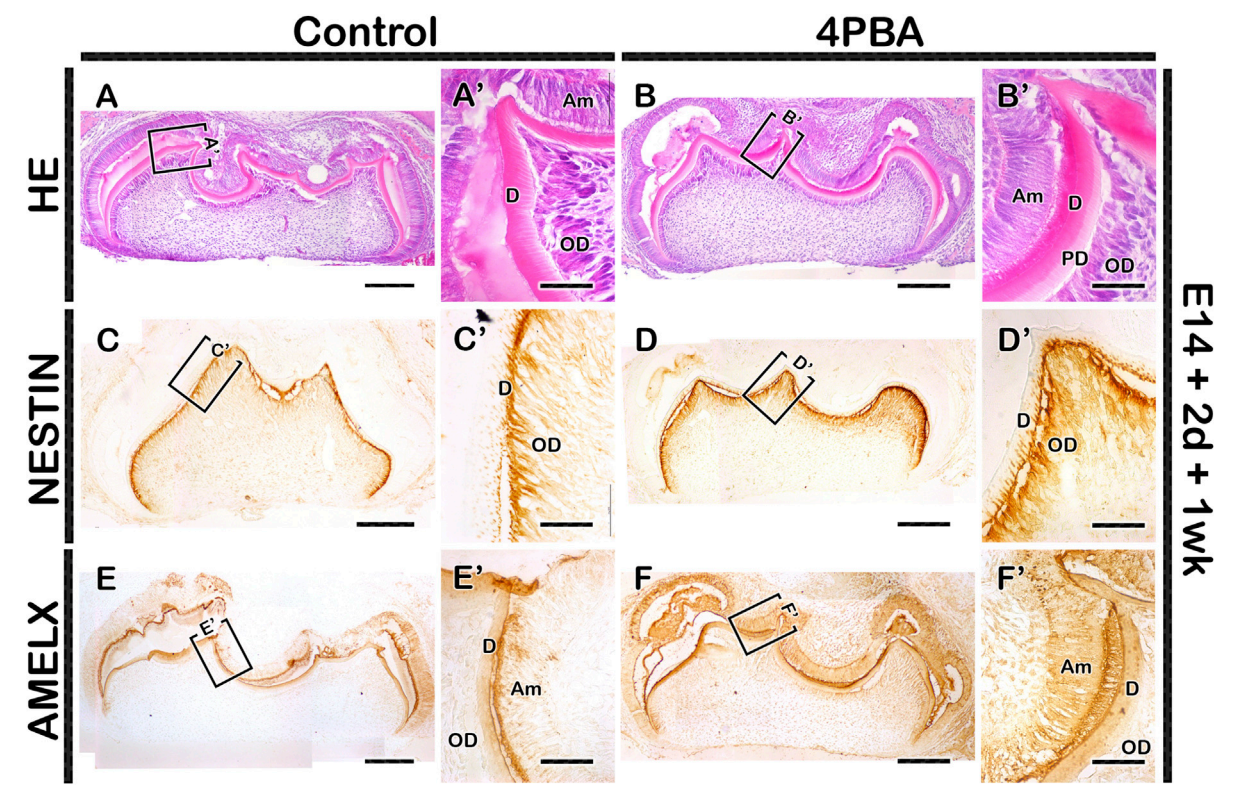

FIGURE 4
Histology and localization patterns of NESTIN and AMELX in renal calcified teeth. HE staining showing comparably thicker enamel and dentin layers in the 4PBA treated teeth compared to controls **(AA',BB')**. Similarly, the localization patterns of NESTIN and AMELX are stronger in the 4PBA treated teeth when compared to control group **(CC'–FF')**. Boxes indicate enlarged views **(A–F)**. Am, ameloblast; OD, odontoblast; PD, predentin; D, dentin. Scale bars: 200 μm **(A–F)**, 50 μm **(A'–F')**.

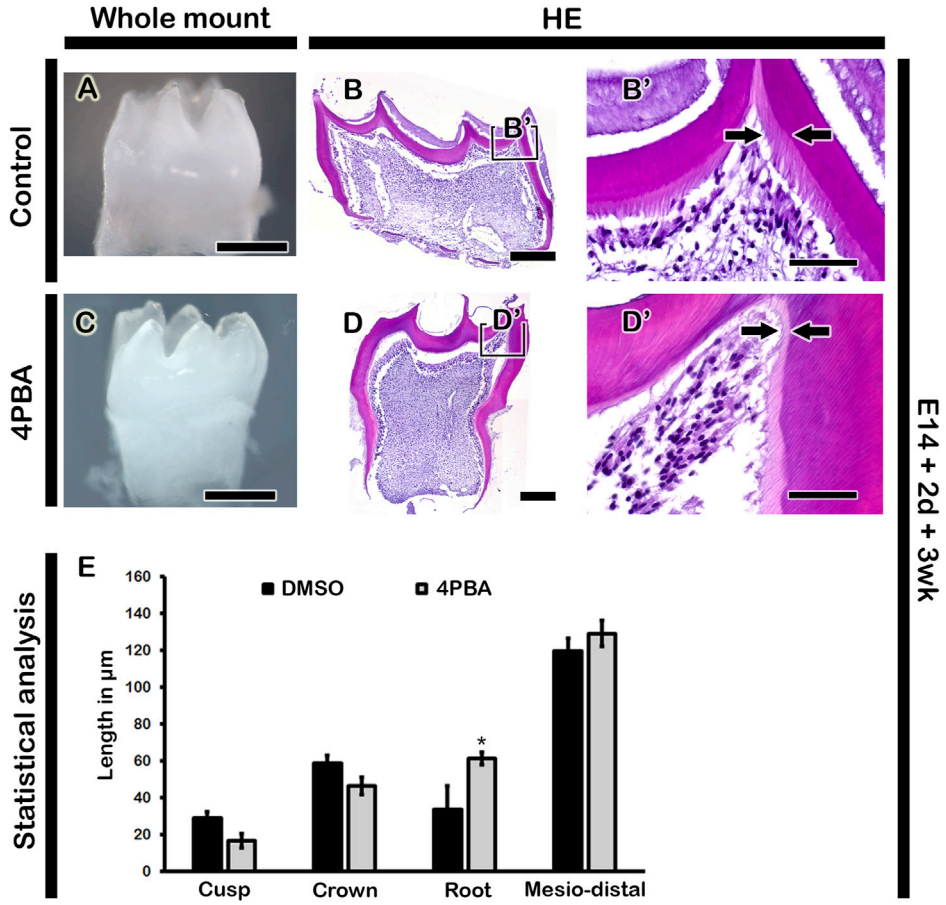

FIGURE 5
Morphology and histology of renal calcified teeth. The 3-week renal calcified teeth showing elongated tooth roots in the 4PBA treated group when compared to control **(A, C)**. Thicker predentin in the cuspal area in the 4PBA treated teeth **(B', D')**. Boxes in B and D indicate enlarged views. Arrows indicate thickness of predentin **(B', D')**. Scale bars: 500 µm **(A, C)**, 200 µm **(B, D)**, 50 µm **(B', D')**. Statistical analysis showing cusp, crown, root and mesio-distal lengths in the control and 4PBA treated group **(E)**. N = 5. * indicates $p < 0.05$.

secretory function, but it appears to inhibit ameloblast apoptosis itself (Brookes et al., 2014). In our study, after treatment of 4PBA, localization of ER stress markers: GRP78 and HRD1, as well as NESTIN and AMELOGENIN were increased with denser alignment of odontoblasts and thicker dentin/enamel space (Figures 3, 4). These results suggest that chemical chaperone (4PBA) facilitates matrix formation in normal tooth development, and as a result of extensive secretion of matrices, ER stress was increased than in normal developing condition. Moreover, alteration in tooth phenotype was due to alteration in cellular events such as proliferation, apoptosis and ER stress in the 4PBA treated teeth (Figures 2, 3) because the alteration in enamel knot signaling play a vital role for future cusp, crown and root morphogenesis (Cho et al., 2007; Aryal et al., 2020; Neupane et al., 2020). The longer root length and thicker matrices of predentin in the 4PBA treated specimen suggest that this chemical chaperone facilitates the transcription of genes during development of tooth root and secretion of matrices because 4PBA as a histone deacetylase inhibitor can possibly facilitate transcription of multiple genes which regulate apoptosis and promote survival in the formation of matrices (Duncan et al., 2013; Brookes et al., 2014; Zhang and Zhong, 2014; Chotikatum et al., 2018).

The application of 4PBA as a drug repositioning method in dental procedures has been practiced for dentin regeneration (Lee et al., 2022). Based on these results, we speculated that proper amount of concentrated drug, 4PBA would affect tooth development in fetal period. The *in vitro* organ cultivation using embryonic stage tooth may imitate human oral environment of tooth transition period because permanent teeth in human initiate to develop in the jaws at birth and continue after a child is born. Our results suggest that direct absorption of chemical chaperone in oral environment during normal primary tooth development may affect matrix formation of enamel and dentin. Additionally, we speculated

that disorders like dentin dysplasia type I which is characterized by short or absence of tooth roots, obliterated pulp chambers (Malik et al., 2015) would be improved through exposure of chemical chaperone, such as 4PBA during permanent tooth development period.

In summary, ER stress occurs inevitably during secretory stage of tooth development. When protein folding capacity is overloaded, the UPR is activated which functions as a significant factor for normal tooth development. Our results showed the alterations in cellular physiology and morphology of developing tooth when treated with chemical chaperone, 4PBA. These results imply that when 4PBA is treated during tooth development period, or specifically during human tooth transition period, there would be both positive and negative effects of chemical chaperone. Further studies about these effects need to be proceeded in the near future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by KNU 2020-0107.

Author contributions

E-SL, YA, J-HH and J-YK contributed to conception, design, and data interpretation, critically revised the manuscript. T-YK, EP, J-YK, HY, C-HA, S-YA, YL, W-JS and J-KJ contributed to data analysis, interpretation, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.1079355/full#supplementary-material

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